Biomedicine & Pharmacotherapy 167 (2023) 115564

Contents lists available at ScienceDirect

Biomedicine & Pharmacotherapy

journal homepage: www.elsevier.com/locate/biopha

Review

Combatting resistance: Understanding multi-drug resistant pathogens in intensive care units

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ARTICLE INFO

Keywords:
Nosocomial infections
Multi-drug resistant opportunistic pathogens
ESKAPE pathogens
Prevalence situation
Resistance mechanisms
Therapeutic strategies

ABSTRACT

The escalating misuse and excessive utilization of antibiotics have led to the widespread dissemination of drug-resistant bacteria, posing a significant global healthcare crisis. Of particular concern is the increasing prevalence of multi-drug resistant (MDR) opportunistic pathogens in Intensive Care Units (ICUs), which presents a severe threat to public health and contributes to substantial morbidity and mortality. Among them, MDR ESKAPE pathogens account for the vast majority of these opportunistic pathogens. This comprehensive review provides a meticulous analysis of the current prevalence landscape of MDR opportunistic pathogens in ICUs, especially in ESKAPE pathogens, illuminating their resistance mechanisms against commonly employed first-line antibiotics, including polymyxins, carbapenems, and tigecycline. Furthermore, this review explores innovative strategies aimed at preventing and controlling the emergence and spread of resistance. By emphasizing the urgent need for robust measures to combat nosocomial infections caused by MDR opportunistic pathogens in ICUs, this study serves as an invaluable reference for future investigations in the field of antibiotic resistance.

1. Introduction

Nosocomial infections (NIs) are a significant concern within healthcare facilities, affecting patients, healthcare workers, and staff. These infections not only result in severe health complications but also pose substantial challenges to the healthcare system. Unfortunately, the prevalence of NIs has been increasing globally. In developed countries, the incidence rate of NIs ranges from 5% to 10%, and around 7% of hospitalized patients experience NIs. In particular, these two sets of data are arousing more attention in developing countries, respectively 20~25%, and approximately 10% [1,2].

In intensive care units (ICUs), infections serve as important causes of morbidity and mortality, particularly due to the heightened vulnerability of ICU patients to NIs. Among these infections, lower respiratory tract infections, including ventilator-associated pneumonia, are the most prevalent in ICUs, with incidence rates ranging from 5% to 40% and an estimated mortality rate of 13% [3]. Urinary tract infections (UTIs) and bloodstream infections (BSIs) rank as the second and third most common NIs in ICUs, respectively. Catheter-associated UTIs contribute to an estimated 59–86% of all hospital-acquired UTIs, with an incidence rate of approximately 54% [4]. Central line-associated BSIs are also frequent healthcare-associated infections, with a meta-analysis reporting an incidence rate of 45.9% [5]. And these opportunistic pathogens that cause severe infections in ICUs mostly come from ESKAPE pathogens.

Notably, opportunistic pathogens, especially in these ESKAPE pathogens including *Enterococcus faecium* (*E. faecium*), *Staphylococcus aureus* (*S. aureus*), *Klebsiella pneumoniae* (*K. pneumoniae*), *Acinetobacter baumannii* (*A. baumannii*), *Pseudomonas aeruginosa* (*P. aeruginosa*), and *Escherichia coli* (*E. coli*), augmented by multi-drug resistance (MDR),

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https://doi.org/10.1016/j.biopha.2023.115564

Available online 23 September 2023
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already have the capacity for aggressive outbreaks and force the closure of ICUs [6]. The widespread and inappropriate use of antibiotics has exerted significant selective pressure on bacteria, leading to the development of resistance mechanisms to evade the lethal effects of these drugs. Consequently, the clinical incidence of antibiotic-resistant bacterial isolates has been maintained at a high level. Fig. 1 shows the detection rates of drug-resistant ESKAPE pathogens in China, the United States, and Europe from 2018 to 2020. In this urgent situation, it is crucial to conduct a comprehensive analysis of bacterial resistance to guide the rational use of antibiotics and reduce the occurrence of resistant bacterial strains. Therefore, this review focuses on recent advancements in understanding the dissemination and resistance determinants of MDR opportunistic pathogens in ICUs. Furthermore, potential strategies for preventing and controlling antibiotic resistance are discussed, aiming to enhance the prevention and control of MDR opportunistic pathogens in ICUs. This review serves as a valuable reference for future studies on antibiotic resistance.

2. MDR opportunistic pathogens

Due to the serious infection caused by MDR ESKAPE pathogens in ICU, this part mainly introduces ESKAPE pathogens to further understand the prevalence and antibiotic-resistant mechanism of MDR opportunistic pathogens.

2.1. *E. faecium*

Among the 58 identified *Enterococcus* species and subspecies, *E. faecalis* and *E. faecium* are the most clinically significant [7]. While *E. faecium* is more pathogenic, *E. faecalis* exhibits greater drug-resistance, making it the primary cause of vancomycin-resistant *Enterococcus* infections [8]. *E. faecium* is a major culprit in healthcare-associated infections and has shown increasing resistance to vancomycin due to nosocomial transmission. In a national survey conducted in Australia in 2016, nearly 50% of *E. faecium* isolates from BSIs were found to be resistant to vancomycin [9]. In the United States, *Enterococcus* ranks second to *Staphylococcus* in terms of hospital-acquired infections, antibiotic-resistant pathogenic diseases, central catheter-related bacteremia, and hospital-acquired endocarditis [10].

Enterococcal antibiotic resistance can arise from inherent mechanisms present in the species' genomes as well as acquired mechanisms involving the acquisition of new genetic material or sporadic mutations in intrinsic genes. Enterococci are intrinsically resistant to most β-lactams, which is attributable to the expression of low-affinity penicillin-binding proteins (PBPs) designated PBP4 in *E. faecalis* and PBP5 in *E. faecium* [11]. Meanwhile, molecular analysis shows that a number of strains of *E. faecalis* produce β-lactamase, which is identical to that produced by *S. aureus*, in some cases within genetic regions identical to *S. aureus* β-lactamase transposon Tn*551* [7]. Resistance to glycopeptide antibiotics in *Enterococcus* involves the acquisition of van gene clusters, which coordinate and modify peptide synthesis. Modified cell wall precursors exhibit significantly reduced affinity for glycopeptides, leading to impaired binding. VanY, an enzyme, hydrolyzes the terminal D-Ala of normal pentapeptide precursors, rendering them unusable for proper cell wall construction [9,12]. The most common resistance of *E. faecium* to aminoglycoside is encoded by the *aac(6′)-Ie-aph(2″)-Ia* and *aph(3′)-IIIa* [13]. Enterococci acquire and transfer drug resistance-related mobile genetic elements through mechanisms such as plasmids, transposons, and integrons, thus acquiring antibiotic

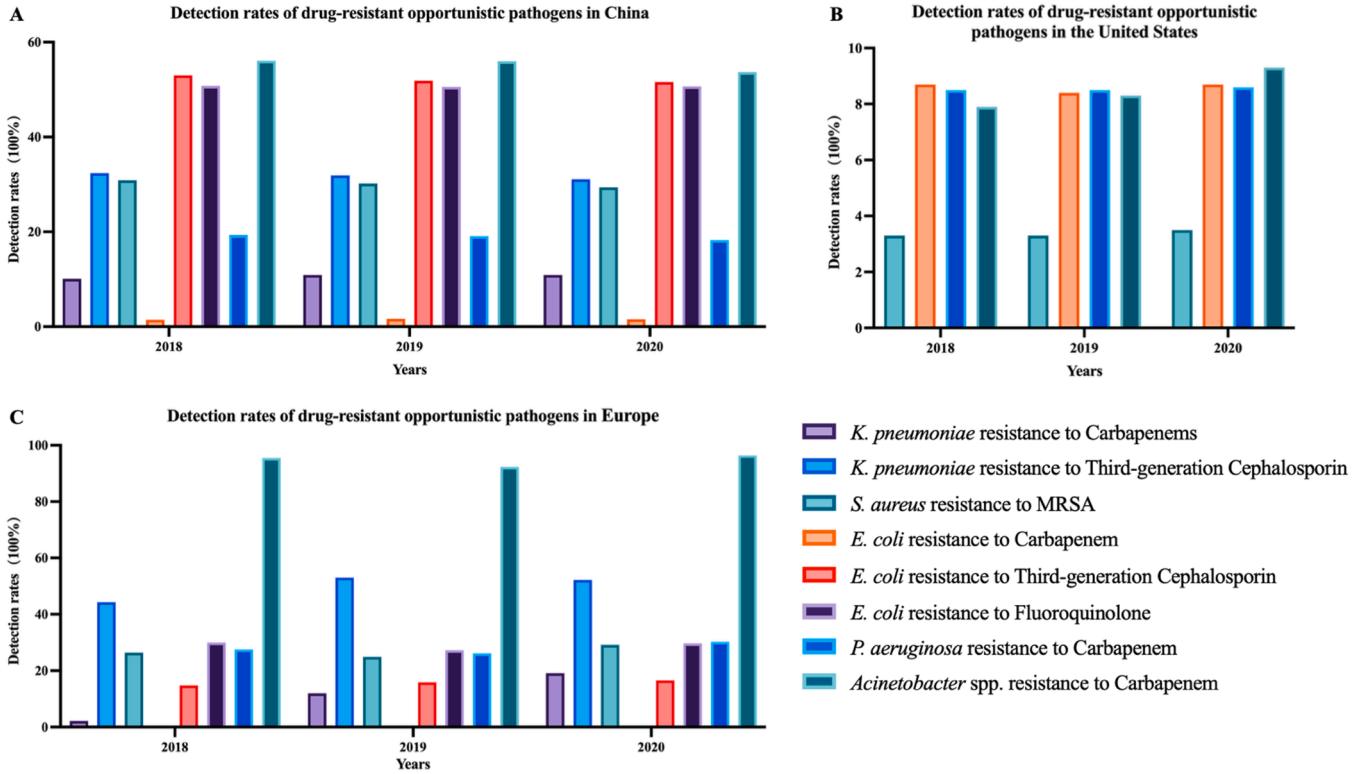
A
Detection rates of drug-resistant opportunistic pathogens in China
Detection rates (100%)
Years
2018
2019
2020
B
Detection rates of drug-resistant opportunistic pathogens in the United States
C
Detection rates of drug-resistant opportunistic pathogens in Europe
K. pneumoniae resistance to Carbapenems
K. pneumoniae resistance to Third-generation Cephalosporin
S. aureus resistance to MRSA
E. coli resistance to Carbapenem
E. coli resistance to Third-generation Cephalosporin
E. coli resistance to Fluoroquinolone
P. aeruginosa resistance to Carbapenem
Acinetobacter spp. resistance to Carbapenem

Fig. 1. Detection rates of drug-resistant ESKAPE pathogens in China, the United States and Europe from 2018 to 2020. (A) Data on common antibiotic resistant strains detected in China comes from the China Two Network Surveillance Cloud (www.carss.cn/sys/Htmls/dist/index.html). (B) Data of United States are collected from COVID-19: U.S. Impact on Antimicrobial Resistance, Special Report 2022. (C) Data of Europe are collected from the Antimicrobial Resistance Surveillance in Europe 2022. The resistance data of pathogens to antibiotics mainly comes from representative drugs of various antibiotics. Carbapenems resistance usually include imipenem and meropenem; The third-generation cephalosporins include cefotaxime, ceftriaxone, ceftazidime; Fluoroquinolones resistance data mainly come from ciprofloxacin, levofloxacin and ofloxacin.

resistance [10]. Horizontal gene transfer between Enterococci occurs through the transfer of pheromone-sensitive or broad-host-range plasmids, as well as the movement of transposons. Pheromone-responsive plasmids are predominantly found in *E. faecalis*, while three types of transposons, namely Tn*3* family transposons, composite transposons, and conjugative transposons, account for most gene movement in *Enterococcus* [12].

2.2. S. aureus

S. aureus is commonly found as a symbiotic flora in the nasal mucosa of 20–40% population [14]. It can be classified into two distinct categories based on its susceptibility to methicillin: methicillin-susceptible *S. aureus* and the more concerning methicillin-resistant *S. aureus* (MRSA), while the latter is considered an opportunistic pathogen. Methicillin resistance is often used as a marker for MDR. Obviously, MRSA strains pose significant challenges in clinical management. Several major MRSA clones have been identified, including CC30, CC45, and CC15 [15]. In the context of MRSA infections, it is noteworthy that the epidemiological landscape may exhibit regional variations. For instance, in China, over 75% of MRSA infections are attributed to three dominant clones: CC239, CC5, and CC59, accounting for 25%, 21%, and 33% of cases, respectively. Similarly, in Europe, the prevailing MRSA clones are CC5-MRSA-II and CC45-MRSA-IV, while in the United States, the most common clone is USA300 [16]. Such regional disparities in MRSA prevalence and clone distribution necessitate tailored strategies for surveillance and infection control.

The mechanisms underlying *S. aureus* drug resistance are intricate and multifaceted. Intrinsic mechanisms of drug resistance include alterations in the external membrane permeability, the presence of efflux pump systems (such as QacA, NorA, and Smr), and the overproduction of β-lactamases. Reduced cell membrane permeability hampers drug absorption and contributes to drug resistance by affecting bacterial energy metabolism. Efflux pump systems play a significant role in MRSA resistance by actively expelling antibiotics from the bacterial cell. MRSA strains also exhibit the enhanced production of β-lactamases, which enzymatically inactivate β-lactam antibiotics through hydrolysis, rendering them ineffective. Additionally, MRSA can tightly bind extracellular antibiotics, impeding their entry into the intracellular space and hindering their target engagement, ultimately leading to antibiotic resistance. Acquired antibiotic resistance mechanisms encompass genetic mutations, acquisition of resistance genes, and biofilm-mediated drug resistance [17]. *S. aureus* can acquire resistance by modifying target DNA gyrase or reducing the genetic expression of outer membrane proteins, thereby limiting drug accumulation. Acquired resistance is often mediated by plasmids, which facilitate the transfer of drug-resistant genes through mechanisms such as plasmid-mediated conjugation, transformation, and insertion. These genetic variations can lead to the overproduction of β-lactamases, contributing to bacterial resistance. Notably, biofilms formed by *S. aureus* exhibit robust adhesion and display enhanced drug resistance, enabling the bacteria to evade host immune responses and evade antibiotic eradication [17]. The formation of biofilms represents a significant challenge in the management of *S. aureus* infections. Understanding the complex mechanisms underlying *S. aureus* drug resistance is crucial for guiding effective treatment strategies. By deciphering the interplay between intrinsic and acquired resistance mechanisms, researchers can develop innovative approaches to combat this formidable pathogen.

2.3. K. pneumoniae

K. pneumoniae, a significant global health threat, has emerged as the most common pathogen of hospital-acquired bacterial pneumonia since 2020[18]. Notably, carbapenem-resistant *K. pneumoniae* (CR-KP) accounts for approximately 60% of carbapenem-resistant *Enterobacteriaceae* (CRE) cases resulting from the excessive use of carbapenem antibiotics [19]. *K. pneumoniae* has demonstrated a remarkable ability to rapidly develop resistance to commonly used antibiotics, reflecting its adaptive response to environmental changes. The prevailing high-risk clones vary across different regions, but the majority of them belong to the ST11, ST15, ST101, ST147, and ST258 categories [20]. ST258 derivatives are widespread CR-KP clones found in Europe and America, while ST11 is frequently encountered in the Asia-Pacific region [21].

In addition, this species usually possesses polysaccharide capsules that play a crucial role in its pathogenesis and evading phagocytosis by the host immune system. In the last two decades, a new species of hypervirulent *K. pneumoniae* (hvKP) have emerged as significant clinical pathogens. Compared to classic *K. pneumoniae* (cKP), hvKP strains typically exhibit high viscosity and overproduce polysaccharide capsules, enabling them to cause highly invasive infections, including liver abscesses, particularly in immunocompromised individuals. Occasional transmission of hvKP has been reported in Europe and the United States. A retrospective study indicated that the prevalence of hvKP clinical isolates with hypermucoviscosity phenotype was only 5.4% in Spain, 8.2% in Canada, and 6.7% in Brazil [22]. However, in South Korea, the infection rate of hvKP was as high as 42.2%, while in China, it ranged from 8.33% to 73.9% [23,24].

The current evidence suggests it has a wider ecological distribution, significantly more varied DNA composition, greater antimicrobial resistance gene diversity, and a higher plasmid burden than other Gram-negative opportunists[25]. *K. pneumoniae* develops various resistance mechanisms to multiple antibiotics (as shown in Fig. 2), including the hydrolysis of modified antibiotics, mutation and transformation of antibiotic target structures, reduced permeability, and overexpression of efflux pumps. The production of extended-spectrum β-lactamases (ESBLs) by *K. pneumoniae* confers resistance to a broad range of β-lactam antibiotics, such as penicillins, cephalosporins, monobactams, and carbapenems [26]. Additionally, mutations in ribosome proteins or 16 S rRNA methylases can result in resistance to aminoglycosides, while mutations in the quinolone resistance-determining region (QRDR) can lead to high-level resistance to quinolones. Reduced permeability can arise from mutations or alterations in the quantity, structure, and quality of outer membrane porin proteins (Omps), resulting in decreased intracellular antibiotic concentration. Lastly, overexpression of multi-drug efflux pumps belonging to the resistance nodulation-division (RND) family can reduce antibiotic accumulation in the cytoplasm, allowing bacteria to adapt to antibiotic stress.

2.4. A. baumannii

A. baumannii, has emerged as a major concern in clinical settings due to the frequent isolation of MDR or extensively drug-resistant strains over the past decade. More recently, pan-drug resistant *A. baumannii* strains have also been identified, posing a significant challenge in healthcare settings [27]. In 2017, the World Health Organization (WHO) listed carbapenem-resistant *A. baumannii* as a top critical priority for which new antibiotics are urgently needed [28]. The prevalent clones of *A. baumannii* exhibit regional specificity. For example, a study conducted in Shanghai identified ST208 as the most prevalent clone (50%), followed by ST191 (21.4%), ST540 (12.5%), ST381 (3.57%), ST643 (3.57%), ST195 (1.79%), and ST368 (1.79%) [29]. In Lebanon, predominant clones include ST1305, ST195, ST218, ST502, and ST2059 [30].

A. baumannii employs several mechanisms to acquire antibiotic resistance, including inherent genetics, chromosomal structural mutations, and horizontal transfer of exogenous genes[31]. Carbapenem-resistance (CR) in *A. baumannii* is primarily achieved through the production of intrinsic or acquired carbapenemase-like β-lactamases. Additionally, resistant mechanisms include the modification of antibiotic target sites, control of antibiotic channels in the bacterial membrane, enzymatic modification of antibiotics, and overexpression of external efflux pump systems. Modifications to

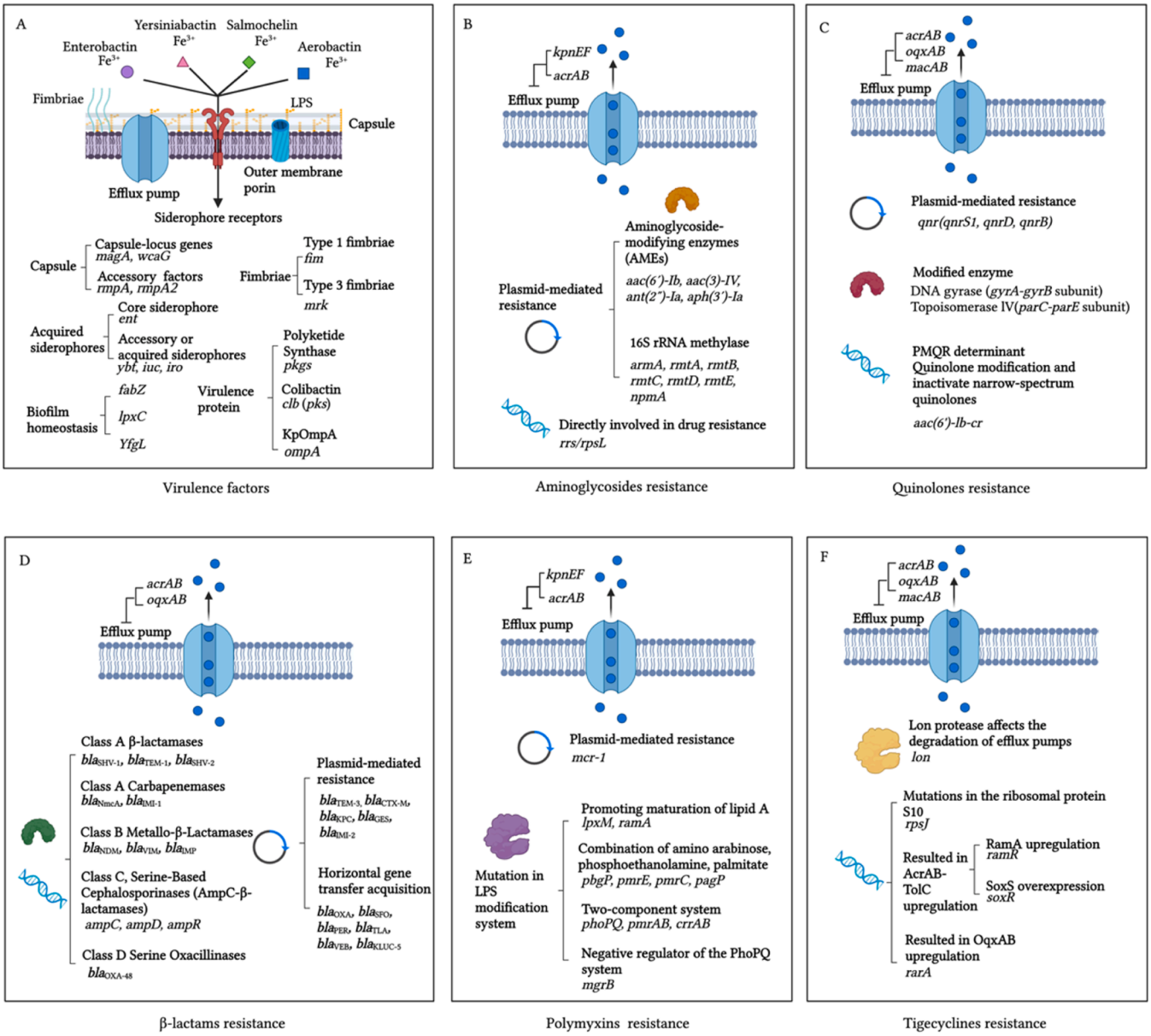

Fig. 2. Virulence factors and Resistance mechanisms of *Klebsiella pneumoniae*. (A) Schematic representation of the main virulence factors of *Klebsiella pneumoniae* and genes of the transcription factors related to virulence factors. The virulence factors include fimbriae, capsule polysaccharides (capsules), LPS and siderophore systems. In addition, a brief list of genes related to biofilm homeostasis is also provided. (B) *K. pneumoniae* possesses a series of genes located in the chromosome or plasmids and encodes specific antibiotic resistance mechanisms. The encoding genes for related proteins are provided in detail in this figure. Resistance to aminoglycosides is mainly due to the production of aminoglycoside-modifying enzymes and 16 S rRNA methylase. (C) Mutations in ribosome proteins or 16 S rRNA methylase can result in resistance to aminoglycosides, especially in quinolone resistance-determining region (QRDR). (D) The production of extended-spectrum β-lactamases (ESBLs) confers resistance to a broad range of β-lactam antibiotics. (E) Mutation in LPS modification system and gene *mcr-1* from plasmid causing resistance to polymyxins. (F) The resistance to tigecycline is mainly due to upregulation and attenuated degradation of efflux pumps, as well as mutations in the ribosomal protein S10.

antibiotic targets can occur through gene mutations or post-transcriptional translation, while antibiotics can be directly inactivated through hydrolysis or modification. *A. baumannii* can reduce membrane permeability or enhance antibiotic efflux to achieve resistance [32]. The low permeability outer membrane protein A (OmpA, 40 kDa) primarily serves a structural component and may play a role in antibiotic transfer from the surrounding environment [32]. Enzymatic modification is the most common mechanism of aminoglycoside resistance in *A. baumannii*. Three types of enzymes, namely acetyltransferase, adenylate transferase, and phosphotransferase, play a key role in aminoglycoside resistance [33]. Overexpression of the AdeABC efflux pump is associated with resistance to carbapenems and cephalosporins in *A. baumannii*, and it is regulated by the AdeRS two-component system. Point mutations in the *adeRS* operons can lead to increased pump expression, resulting in the antibiotic resistance [32].

2.5. *P. aeruginosa*

P. aeruginosa, is a common nosocomial pathogen frequently associated with UTIs and BSIs acquired in ICUs. Surveillance data from the China Antimicrobial Surveillance Network (CHINET) reported that the antibiotic resistance rates of imipenem, ceftazidime, ciprofloxacin, piperacillin/tazobactam and amikacin of *P. aeruginosa* were respectively 23.6%, 23.4%, 14.8%, 13.4% and 6.1% in 2017 [34]. In the United

States, the detection rate of quinolone-resistant *P. aeruginosa* in hospitals exceeded 30% [35]. A study by Giani et al. found that 60% of *P. aeruginosa* clinical isolates collected from 20 medical centers were resistant to at least one β-lactam antibiotic, with 37.2% displaying a MDR phenotype in Italy [36]. *P. aeruginosa* exhibits diverse sequence types (STs), and international high-risk clones such as ST235, ST111, ST175 and ST233 are frequently associated with carbapenemase production [37].

P. aeruginosa possesses intrinsic resistance mechanisms, including reduced outer membrane permeability, inducible production of cephalosporinases, and efflux pumps. Modifications to and decreased expression of the OprD pore protein contribute to reduced drug uptake, resulting in resistance to imipenem. The bacterium also harbors genes encoding AmpC β-lactamases, conferring resistance to β-lactam drugs such as cefuroxime and ceftriaxone. Overproduction of AmpC can arise from mutations in regulatory genes. *P. aeruginosa* possesses four robust RND multidrug-resistant pumps (Mex) that actively extrude toxic compounds from the periplasm and cytoplasm, especially the MexAB-OprM and MexXY-OprM efflux pumps, which play a crucial role in intrinsic resistance. Overexpression of MexXY-OprM in *P. aeruginosa* confers resistance to aminoglycosides, fluoroquinolones, and cephalosporins. Moreover, many clinical *P. aeruginosa* isolates express MexCD-OprJ and MexEF-OprN efflux systems [38]. *P. aeruginosa* can also acquire additional resistance through point mutations in pre-existing genes or the horizontal transfer of resistance determinants.

2.6. *E. coli*

Enterobacterium bacteria, especifically *E. coli*, are Gram-negative bacteria belonging to the *Enterobacteriaceae* family. While *E. coli* normally colonizes the human intestine without causing harm, it can opportunistically lead to severe extraintestinal infections, including bacteremia and sepsis. The MDR ST38 *E. coli* strain exhibits resistance to at least 15 antibiotics. Approximately 70% of clinical *E. coli* infections are resistant to fluoroquinolones (FQs), and 55% are resistant to both FQs and trimethoprim-sulfamethoxazole [39]. The prevalent clonal lineages of MDR *E. coli* include ST131, ST393, ST69, ST95, ST38, and ST73. Among these, ST131 accounts for approximately 70% of clinical cases, making it the most common cause of parenteral infections [40].

Drug resistance mechanisms in *Enterobacteriaceae* involve defects in pore proteins, increased expression of efflux pumps, and mutations in antibiotic targets. The bacterial family possesses general non-specific pore proteins, including Omp35, Omp36, and Omp37, along with specific pore proteins like LamB and PhoE, which resemble outer membrane proteins found in *E. coli*. These channels facilitate the entry of antibiotics through the outer membrane, aiding their accumulation in the periplasmic space. The transcription, translation, and assembly of pore proteins are regulated by a two-component system (TCS) pathway. The AcrAB-TolC pump complex, found in *Enterobacteriaceae* such as *E. coli*, is responsible for effluxing antibiotics. The expression of pore proteins and resistance-nodulation-division (RND) efflux complexes can be regulated by the MarR regulator, which can be further influenced by global regulators like SoxS or Rob [41]. Overexpression of the AcrAB efflux pump and reduced expression of pore proteins are characteristic features of aminoglycoside-resistant MDR strains of *E. coli* [38]. In *Enterobacteriaceae*, mutations in the QRDR within target enzymes, such as gyrases or topoisomerases, are commonly found and confer high levels of quinolone resistance in clinical isolates [41]. Addtionally, polymyxin resistance in *E. coli* can be attributed to chromosomal mutations or the presence of plasmid-borne resistance genes.

Opportunistic pathogenic bacteria have developed various mechanisms of drug resistance, including antibiotic inactivation or modification, alterations in target sites such as enzymes, ribosomes, and cell wall components. Reduced intracellular drug accumulation can occur due to loss of pore proteins or overexpression of external efflux pumps. Biofilm formation provides a mechanical and biochemical barrier, making it challenging for conventional antibiotics to penetrate. The biofilm matrix creates an environment with low oxygen, low pH, high CO_2 levels, and limited water availability, which further contributes to the difficulty in eliminating bacteria [38].

3. Resistance mechanisms of MDR opportunistic pathogens

Antimicrobial drugs, encompassing antibiotics, antifungal drugs, antiviral drugs, and antiparasitic drugs, play a vital role in clinical treatments against various infections. In ICUs, polymyxins, carbapenems, and tigecycline are frequently utilized antibiotics to combat MDR bacteria. However, the alarming rise in antibiotic resistance has been fueled by the plasmid-mediated horizontal transfer of mobile-resistant genes, such as *mcr*, bla_{KPC}, *tmexCD-toprJ*, and *tet*(X4), among the microbiota. This phenomenon has caused a malignant circulation, leading to the significant increase in antibiotic usage, as illustrated in Table 1. This section of the paper aims to provide a comprehensive explanation of the molecular mechanisms underlying MDR in opportunistic pathogens commonly encountered in ICUs.

3.1. *Polymyxins resistance*

Polymyxins are often considered the last line of defense against important MDR Gram-negative clinical isolates. These antimicrobial agents target the lipopolysaccharide (LPS) present on the outer membrane of Gram-negative bacteria. The mechanism of action involves the affinity of the positively charged polymyxin with the negatively charged phosphate groups of the lipid A component of LPS. This interaction leads to the displacement of divalent cations (Ca^{2+} and Mg^{2+}) from the phosphate groups. Consequently, the structural integrity of LPS is compromised, resulting in increased bacterial membrane permeability. The elevated permeability leads to cytoplasmic leakage and ultimately bacterial cell death. Fig. 3 depicts the binding patterns of polymyxins to the target site on Gram-negative bacteria.

Polymyxin resistance primarily arises from the modification of the

Table 1
MIC_{90} of antibiotics against drug-resistant opportunistic pathogens.

Bacterial species	Types of antibiotics	Antibiotics	MIC_{90} (mg/L)	Ref.
K. pneumoniae	Aminoglycosides	Gentamicin	256	[136]
		Tobramycin	64	[136]
		Amikacin	16	[136]
	Glycopeptidess	Vancomycin	≥ 1.0	[137]
	Penicillins	Temocillin	16	[137]
	β-lactams	Cefotetan	2	[138]
		Cefepime-Enmetazobactam	≤ 0.5	[140]
	Polypeptides	Colistin	24	[139]
S. aureus	Tetracyclines	Omadacycline	1.0	[141]
E. coli	Cephalosporins	Cefepime-zidebactam	≤ 4	[140]
		Cefotetan	2	[138]
		Cefepime-Enmetazobactam	≤ 0.5	[140]
	Tetracyclines	Omadacycline	4	[141]
	Carbapenemases	Tebipenem	0.06	[142]
		Mecillinam	> 256	[137]
	Aminoglycosides	Apramycin	8	[143]
Acinetobacter spp.	Aminoglycosides	Apramycin	16	[143]
P. aeruginosa	Monobactams	Aztreonam	32	[144]
	Cephalosporins	Cefepime-taniborbactam	32	[144]
	Quinolones	Ciprofloxacin	32	[144]
	Carbapenems	Doripenem	2	[145]
		Meropenem	2	[145]
		Imipenem	8	[145]

MIC_{90} : minimum inhibitory concentration at which 90% of isolates were inhibited

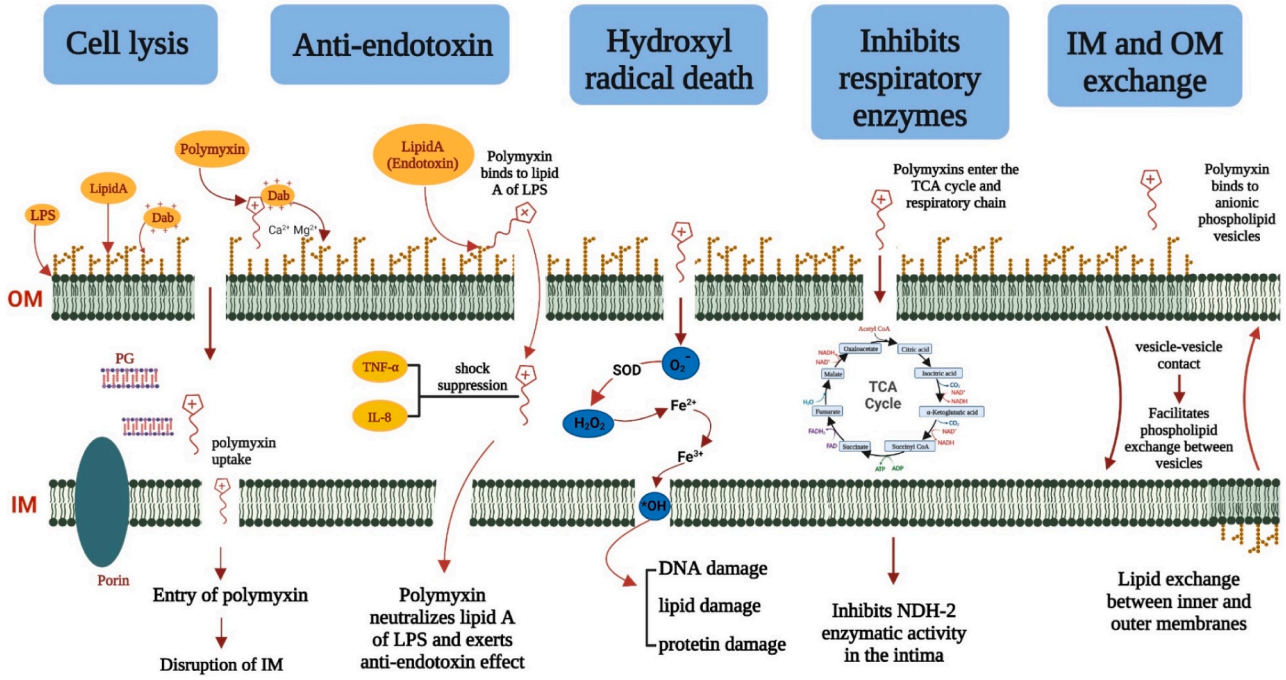

Fig. 3. Antibacterial mechanisms of polymyxins against Gram-negative bacteria. (A) Direct bacteriostatic mechanism: electrostatic interactions between di-amino butyric acid (Dab) of polymyxin residues and anionic phosphate groups on the outer membrane of LPS lipid A allows polymyxin to fuse and interact with the membrane, disrupting the inner and outer membranes and thus causing cell lysis; (B) Endotoxin mechanism: lipid A of LPS is an endotoxin of Gram-negative bacteria. Polymyxin inhibits the activity of endotoxin lipid A by binding and neutralizing LPS, thereby eliminating endotoxin by releasing cytokines such as tumor necrosis factor-α (TNF-α) and Interleukin 8 (IL-8); (C) Hydroxyl radical death pathway: polymyxins are known to damage DNA, lipids and proteins, producing reactive oxygen species (ROS) resulting in cell death; (D) Inhibition of energy-producing enzymes: antibacterial effects of polymyxins are achieved by inhibiting key enzymes for energy production; (E) Vesicle-trafficking contact pathway: after passing through the outer membrane, polymyxins bind to anionic phospholipid vesicles. Therefore, The inner and outer membrane layers links, resulting in phospholipid loss and bacterial cell death. OM: Outer membranes; IM: Inner membranes; PG: Peptidoglycan; SOD: Superoxide dismutase; TCA cycle: Tricarboxylic acid cycle; NDH-2: Type II NADH dehydrogenase.

lipid A component of LPS on the bacterial membrane. This modification involves the addition of 4-amino-4-deoxy-L-arabinose (L-Ara4N), phosphoethanolamine (pEtN), or galactosamine to lipid A. Various genes, operons, and regulatory factors contribute to the qualitative modification of LPS, including *pmrC*, *pmrE*, and the *pmrHFIJKLM* operon, as well as regulatory factors like MgrB (which negatively regulates the PhoPQ system) and the CrrAB TCS that regulates PmrAB [42]. Environmental cues, such as macrophage phagocytosis, exposure to metal ions, and low pH conditions (e.g., pH 5.5), can activate the periplasmic domains of PmrB and PhoQ. These domains possess tyrosine kinase activity, resulting in the phosphorylation of PmrA and PhoP. Subsequently, PmrA and PhoP activate the *pmrHFIJKLM* operon, leading to the modification of LPS through the addition of L-Ara4N and pEtN [43]. PhoP can also directly or indirectly activate PmrA via the PmrD connector pathway [44]. Activation of the PmrAB and PhoPQ systems is typically observed when bacteria are engulfed by macrophages, enabling the bacteria to survive within the host (Fig. 4).

In 2016, Liu et al. reported the discovery of the first plasmid-mediated polymyxin resistance gene, *mcr-1* [45]. Since then, additional genes, including *mcr-2* to *mcr-10*, have been identified. These genes encode MCR family proteins, which are phosphoethanolamine (PEA) transferases that modify the phosphate group of lipid A by adding PEA. This modification reduces the negative charge of the bacterial outer membrane, decreasing its affinity to polymyxins. The structure-acivity analysis has revealed that the MCR family of resistance enzymes share a similar phosphatidylethanolamine (PE) lipid substrate binding cavity and utilize an identical "ping-pong" enzyme catalytic mechanism to confer resistance [46]. It is worth noting that while *mcr-6* is chromosomally localized, the other *mcr* genes are located on plasmids [47]. The presence of transposon elements in plasmids carrying *mcr* genes facilitates their spread into different plasmid backbones, thereby driving the development of polymyxin resistance in Gram-negative bacteria.

The dissemination of *mcr* genes is often mediated by mobile genetic elements, such as insert sequences (IS) or integrons. Serial studies have shown that the transposon Tn*6330*, which contains the IS*Apl1-mcr-1-orf*-IS*Apl1* element, can excise from the plasmid and then form a circular intermediate of IS*Apl1-mcr-1-orf*, which can integrate into other plasmids carrying IS*Apl1* [48]. In the case of *K. pneumoniae*-producing carbapenemase, the plasmid pMCR1-IncX4 was comparatively analyzed, revealing that IS*Apl1* and *mcr* are not always co-present. IS*Apl1* is commonly found in IncHI2-type plasmids and may or may not be present in IncI2-type plasmids, but it is entirely absent in IncX4-type plasmids. One possibility is that *mcr* may have originated from plasmids and subsequently co-evolved with the acquisition of IS*Apl1*, leading to its rapid mobilization to other plasmids. Alternatively, the *ISApl1* transposase may be lost after the translocation of the IS*Apl1-mcr-1* element into the plasmid, forming an IS*Apl1* composite transposon to enhance the stability of *mcr* in the plasmid [49]. The presence of the IS*Apl1-mcr-1* region highlights the importance of IS*Apl1* in the chromosomal integration of *mcr-1*.

In contrast to IS*Apl1*-mediated *mcr-1* translocation, a unique complex transposon containing IS*1595* (renamed IS*Ec69*) has been found to acquire or transfer the *mcr-2* gene [50]. The *mcr-2* gene, when flanked by two identical IS elements, can form cyclic intermediates through homologous recombination, which facilitates the rapid spread of MCR-2 colistin resistance in diverse bacterial hosts. Our group identified the *mcr-3* gene as an inherent resistance determinant [51]. We discovered that the *mcr-3* gene is related to Tn*As2* and Tn*As3*, which are transposons of the Tn*3* family, suggesting the possibility of *mcr-3* transfer from *Aeromonas* species to *Enterobacteriaceae*. Borowiak et al. identified the *mcr-5* gene in polymyxin-resistant *A. baumannii*, where it is

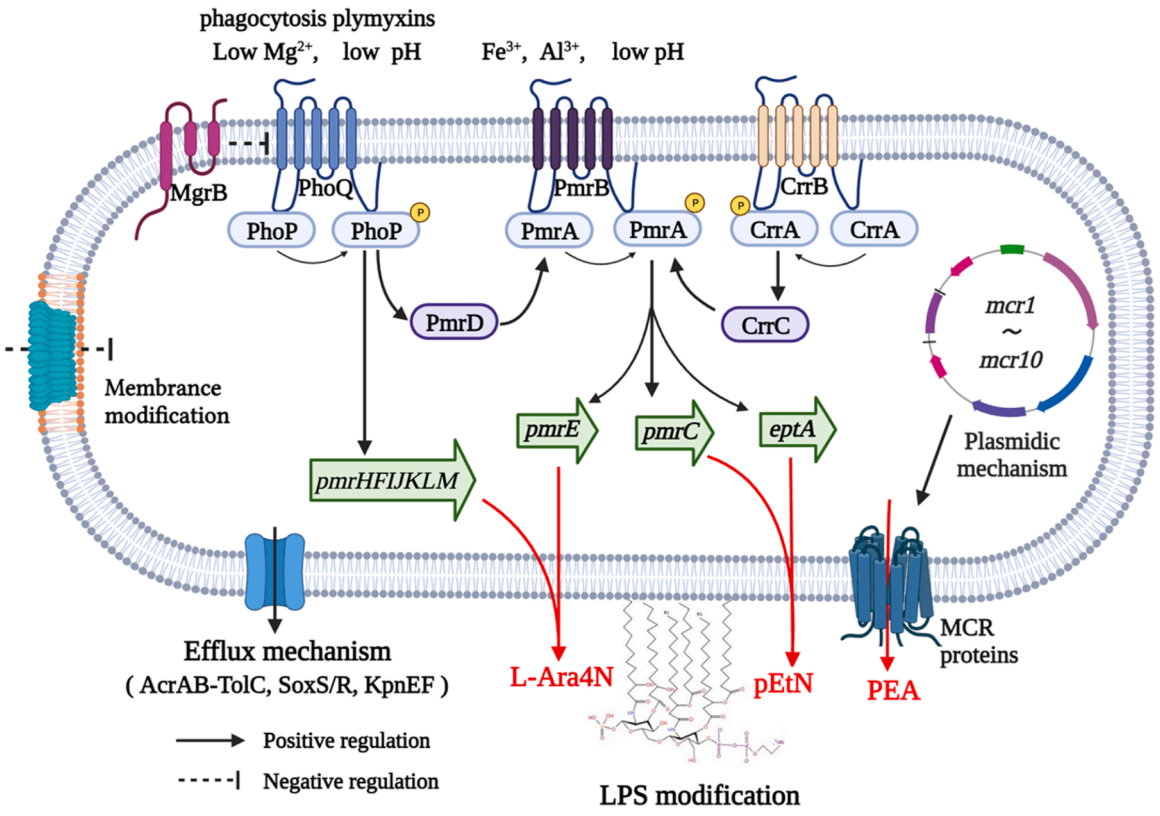

Fig. 4. Mechanisms of polymyxins resistance. The plasmid-mediated polymyxins resistance gene *mcr* encodes pEtN and L-Ara4N is transferred to modify lipid A of LPS. This modification thereby reduces the electrostatic interaction of polymyxins with LPS, leading to bacterial resistance. Activation of the two-component systems PmrAB, PhoPQ and CrrAB, alteration of the barrier system, and enhancement of the broad-spectrum efflux pump system can also confer bacterial resistance to polymyxins. MgrB: Negative regulator of two-component systems.

Table 2
Summarized information of *mcr* genes.

Genes	Related plasmid replicon type	Host bacterial species	Potential source of *mcr* gene	Sequence identity (compared to MCR-1 prototype)	Active site	Ref.
mcr-1	IncI2, IncX4, IncHI2/HI2A, IncHI1, IncN, IncP, IncQ, IncY, IncPO111, IncX1-X2, IncX2, IncX3-X4, IncFI, IncFII	*Escherichia coli, Klebsiella pneumoniae, Salmonella, Enterobacter, Shigella, Citrobacter, Moraxella*	*Moraxella pleuranimalium*	100%	E246, T285 H395, D465, H466	[45,146]
mcr-2	IncX4	*Escherichia coli, Salmonella, Moraxella*	*Moraxella pleuranimalium*	81%	E244, T283, H393, D463, H464, H476	[146]
mcr-3	IncHI2, IncP, IncF	*Escherichia coli, Klebsiella pneumoniae, Salmonella, Shigella, Proteus mirabilis, Aeromonas*	*Aeromonas* spp.	33%	E240, T278, H377, D452, H453	[147]
mcr-4	ColE	*Shewanella, Enterobacter cloacae, Escherichia coli, Salmonella, Acinetobacter*	*Shewanella frigidimarina*	34%	E240, T278, H377, D452, H453, E248	[148]
mcr-5	ColE, IncX1	*Enteric Streptococcus, Escherichia coli, Pseudomonas aeruginosa, Aeromonas hydrophila*	*Legionella, Burkholderiales*	36%	T286, H389, D458, H459	[149]
mcr-6	NA	*Moraxella*	*Moraxella pluranimalium*	83%	NA	[150]
mcr-7	IncI2	*Klebsiella pneumoniae*	*Aeromonas* spp.	35%	NA	[149, 151]
mcr-8	IncFII	*Klebsiella pneumoniae, Raoultella* spp., *Stenotrophomonas*	*Stenotrophomonas*	*33%*	E246, T285 K333, H395, D465, H466, E468, H478	[47,53, 149]
mcr-9	IncFII, IncHI2A, TrfA, IncHI2	*Salmonella, Klebsiella, Enterobacter, Salmonella, Leukella, Citrobacter, Raoultella* spp., *Raoultella ornithinolytica*	*Cronobacter , Enterobacter , Shewanella, Buttiauxella*	36%	NA	[47,54, 149, 152]
mcr-10	IncFIA, IncFIB-FII, IncFIB	*Citrobacter, Enterobacter, Escherichia, Klebsiella, Kluybacterium, Raoultella* spp.	*Buttiauxella*	*29%*	E238, T277, K325, H380, D450, H451, E453, H463	[55,153]

spp.: species; NA: Not Available.

embedded in a Tn*3* family transposon encoding a resolvase and a transposase*3* [52].

In the case of *mcr-7*, no mobile elements were identified in the flanking regions, indicating that it may have become immobilized on plasmids following the loss of flanking IS elements. Studies on *mcr-8* have revealed the presence of two complete IS*903B* inserts upstream and downstream of the gene, as well as the complete insert IS*Ecl1* downstream of *mcr-8*. This suggests that these IS elements (IS*903B*, IS*Ecl1*) may play a crucial role in the dissemination of *mcr-8* among *Enterobacteriaceae* [53]. Whole-genome sequencing has shown that *mcr-9* is located on an IncHI2A-type plasmid, with flanking regions surrounded by IS*903B* and IS*26* [54]. Notably, studies by Wang et al. have indicated that *mcr-9* does not confer polymyxin resistance, suggesting that its transfer may not significantly contribute to the spread of colistin resistance [54]. The *mcr-10* gene, detected in a drug-resistant isolate, was found upstream adjacent to the *xerC* gene, which encodes a tyrosine recombinase involved in the mobilization of genetic elements through site-specific recombination. This suggests that site-specific recombination mediated by XerC may be a common mechanism for the mobilization of *mcr-10*. The identification of various IS sequences downstream of *xerC-mcr-10*, such as IS*Ec36*, suggests that this region may serve as a hotspot for the insertion of mobile genetic elements [55]. A brief summary of *mcr* genes can be found in Table 2.

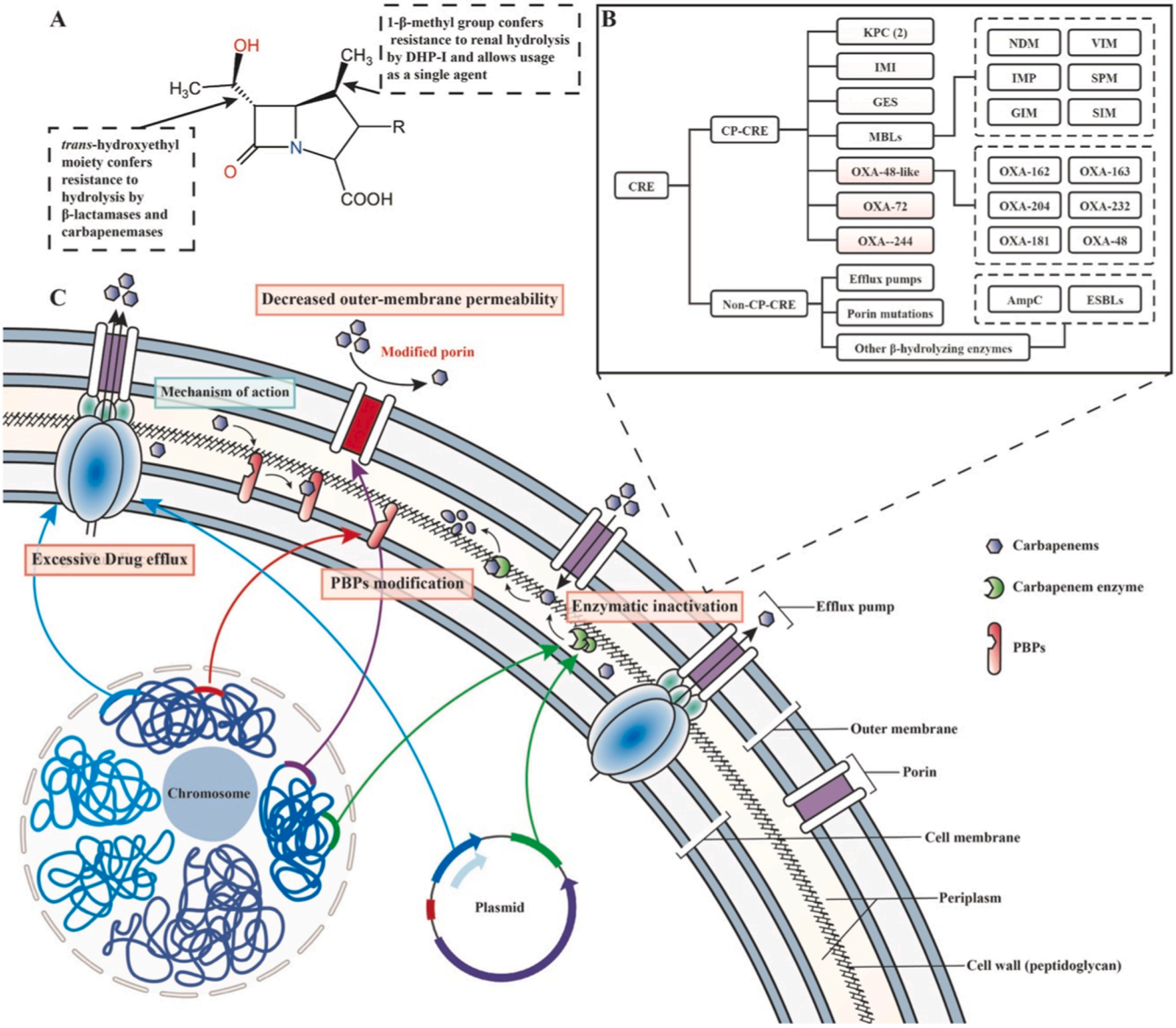

Fig. 5. Mechanisms of carbapenems resistance. (A) Schematics of critical structural properties for carbapenems; (B) Work model of CR represented by Gram-negative bacteria (Green boxes represent mechanisms of drug action, and red boxes represent mechanisms of resistance). Carbapenems act by inhibiting cell wall mucopeptide synthetase, hindering cell wall mucopeptide synthesis and causing bacterial cell wall defect, individual expansion, further leading to changes in cytoplasmic osmotic pressure and cell lysis. The mechanisms of CR can be summarized into four categories: (1) decreased outer membrane permeability through production of modified porins, loss of porin expression, or a shift in the types of porins found in the outer membrane; (2) the antibiotic is pumped out via the specific efflux transporter; (3) drug target modification, such as a mutation in the PBPs; (4) antibiotics are inactivated by chromosome- and/or plasmid-encoding enzymes that hydrolyze β-lactams. (C) The classified genes of drug resistance in CRE (light grey: Ambler Class A, white: Ambler Class B, light red: Ambler Class D). CR: Carbapenem resistance; PBPs: Penicillin-binding proteins; CRE: Carbapenem-resistant *Enterobacteriaceae*; KPC, IMI, GES: carbapenemase (Ambler Class A); MβL: Metallo-β-lactamase (Ambler Class B); OXA-48-like: Serine Oxacillinases (Ambler Class D); OXA-72 and OXA-244: Carbapenemase obtained through horizontal gene transfer; AmpC: Serine-based Cephalosporinases (Ambler Class C); ESBL: Extended-spectrum β-lactamases.

3.2. Carbapenems resistance

Opportunistic pathogens with carbapenems resistance (CR) commonly encountered in ICUs are a significant concern. These pathogens, including *K. pneumoniae*, *E. coli*, *A. baumannii*, and *P. aeruginosa*, exhibit resistance to cephalosporins, carbapenems, aminoglycosides, and fluoroquinolones (Fig. 5A). The resistance phenotype of CRE can be classified into two main sub-groups: non-carbapenemase-producing CRE (non-CP-CRE) and carbapenemase-producing CRE (CP-CRE). In Gram-negative bacteria, the production of β-lactam hydrolases, such as β-lactamases, is a common mechanism for inactivating penicillins. Over time, with the widespread use of β-lactam antibiotics, the emergence of plasmid-encoded ESBLs and AmpC cephalosporinases has become increasingly prevalent. These enzymes are capable of hydrolyzing a broad range of β-lactam antibiotics, including cephalosporins. While they can contribute to resistance to these antibiotics, they are not always associated with CR.

CR is a significant challenge in the field of biomedical research. Fig. 5B illustrates the mechanisms involved in CR. Among these mechanisms, the production of antibiotic hydrolases plays a crucial role. The majority of CR cases can be attributed to the presence of specific enzymes, namely *K. pneumoniae* carbapenemase (KPC, Ambler Class A), Metallo-β-lactamase (MβL, Ambler Class B), and OXA-48-like carbapenemase (Ambler Class D). In China, an alarming survey conducted revealed that 97.4% of CRE strains were found to produce carbapenemases. The most prevalent carbapenemases identified were KPC-2 (51.6%), NDM (35.7%), and OXA-48-like carbapenemase (7.3%) [56]. Similarly, in Canada, the common carbapenemases produced by CRE strains were KPC (66.9%) and NDM-1 (17.3%) [57]. Conversely, in Sudan, 58.7% of CRE strains were found to carry one or more carbapenemase genes. The bla_{NDM} type was the predominant gene, accounting for 52% of cases, followed by bla_{IMP} (3.4%) and bla_{VIM} (0.9%) [58]. In addition to carbapenemase enzymes, another important aspect of CR involves the presence of PBPs. PBPs are a group of enzymes, such as transpeptidase, carboxypeptidase, and endopeptidase, which are located on the inner membrane of bacterial cells. These enzymes play a critical role in the synthesis of the cell wall, forming linear polymers that provide structural integrity to the bacteria [59]. Carbapenems exert their potent bactericidal effects by binding tightly to PBPs, thereby inhibiting the activity of transpeptidase. However, mutations in the gene encoding PBPs can lead to alterations in the number or conformation of these proteins, resulting in decreased binding affinity to carbapenems. Consequently, the effectiveness of carbapenems in eradicating bacterial infections is compromised. It is crucial to understand and address the mechanisms underlying CR to develop effective strategies for combating these drug-resistant pathogens.

Non-CP-CRE resistance involves additional mechanisms such as drug efflux pumps and alterations in the permeability of the outer membrane. The outer membrane acts as a barrier that antibiotics must traverse to enter bacterial cells and exert their bactericidal effects. Reduced expression or absence of outer membrane proteins can impede antibiotic penetration, resulting in increased minimum inhibitory concentration values for carbapenems. When coupled with ESBLs or AmpC enzymes, a synergistic effect can lead to high levels of resistance. Overexpression of efflux pump families can simultaneously confer multidrug resistance to β-lactams, quinolones, and aminoglycosides [60]. Notably, the AdeABC efflux pump system plays a significant role in CR in *A. baumannii*, while *P. aeruginosa* achieves resistance to meropenem through the overexpression of MexAB-outer membrane porin M (OprM), MexCD-OprJ, and MexXY-OprM efflux systems (Fig. 5C).

In 2014, the WHO reported the global spread of carbapenem-resistant *K. pneumoniae* (CR-KP), resulting in substantial morbidity and mortality. CHINET data indicates a more than 8-fold increase in CR rates of *K. pneumoniae* from 2005 to 2020 (from 3.0% to 24.2%) [46]. The production of KPC is the dominant resistance mechanism in CR-KP. KPC enzymes can hydrolyze all β-lactam antibiotics, and strains carrying the bla_{KPC} genes often exhibit resistance to fluoroquinolones, aminoglycosides, and other antibiotics, resulting in MDR. Among CRE strains isolated from both adult and pediatric patients, the most prevalent carbapenemase gene is bla_{KPC-2} (51.6%) [56]. Genetic analysis has revealed that bla_{KPC-2} is typically flanked by IS*Kpn8* (upstream) and IS*Kpn6* (downstream), along with various mobile genetic elements such as IS*26*, IS*6100*, Tn*3*, and *tnpR* located further upstream, as well as IS*26*, Tn*1403,* and *tnpR* downstream [61]. Furthermore, the bla_{KPC-2} gene can spread through bacterial plasmids belonging to incompatible groups IncF, IncN, IncL/M, and IncX [62], thereby exacerbating the progressive deterioration of CR among opportunistic pathogens in clinical settings.

The dissemination of MβL is closely related to horizontal transfer. Currently, MβL genes are carried on mobile genetic elements such as IMP, VIM, SPM, GIM, and NDM [63]. The identification of NDM-1 in *K. pneumoniae* and *E. coli* in 2009 marked the beginning of its rapid spread among other Enterobacteriaceae, posing a global health threat [64]. Within *Enterobacteriaceae*, there are 20 different replicon types of plasmids that carry bla_{NDM}, with IncX3 and IncC (IncA/C) being the most prevalent [65]. The insertion sequence IS*Aba125* is located upstream of bla_{NDM} and provides the promoter region for the expression of bla_{NDM-1} [66]. The original mobilization of the bla_{NDM-1} gene could have occurred through a rolling-circle transposition process involving IS*CR21*, and mobilized it together with MβL genes, *iso* (phosphoribosyl anthranilate isomerase), *tat* (twin-arginine translocation pathway signal sequence protein), *dct* (divalent cation tolerance protein), *groES-groEL* to the downstream IS*Aba125*, forming the gene cassette accounting for the bla_{NDM-1} resistance. A second copy of IS*Aba125* is then inserted downstream of IS*CR21*, forming the IS*Aba125*-based complex transposon Tn*125* [67].However, recent studies have found that Tn*125* can be replaced by other elements, such as the pseudo-composite transposon flanked by IS*26* and Tn*3000* [65]. The spread of bla_{NDM} involves multiple mobile genetic elements, facilitating the widespread transmission of this gene between different plasmids or plasmids and chromosomes. Similar to KPC, NDM expression is associated with varying levels of CR, often accompanied by other resistance determinants, including genes encoding other β-lactamases and carbapenemases, as well as genes associated with fluoroquinolone and aminoglycoside resistance [68].

Since the first identification of OXA-48, multiple variants differing in the substitutions or deletions of amino acids have been reported. OXA-48-like enzymes, including OXA-162, OXA-163, OXA-181, OXA-204, and OXA-232, exhibit very similar resistance patterns. OXA-163, which has a single amino acid substitution and four amino acid deletions compared to OXA-48, only slightly reduces the efficacy of cephalosporins and carbapenems [69]. The propagation of bla_{OXA-48} is primarily driven by the complex transposon Tn*1999*, and its variants are located on the pOXA-48a-like IncL plasmid. The pOXA-48a is highly transferable, with a frequency 50-fold higher than that of another IncL plasmid pNDM-OM bearing bla_{NDM-1}. This high transferability could result from the insertion of the Tn*1999* variant and disruption of the *tir* region of pOXA-48a, whereas the *tir* gene encodes a protein that inhibits dissemination. The conjugation of pOXA-48a-like IncL plasmids mediates the transmission of resistant genes among several bacterial species, including *E. coli*, *K. pneumoniae*, *Enterobacter cloacae*, *K. oxytoca,* and *Citrobacter fowleri* (*C. fowleri*).

3.3. Tigecycline resistance

Tigecycline, an emerging tetracycline antibiotic, inhibits protein synthesis by binding reversibly to the bacterial ribosome 30 S subunit, thereby blocking the entry of tRNA into the amino acyl-tRNA (ribosomal A) site. Unfortunately, a growing number of clinically relevant tigecycline-resistant strains have limited the effectiveness of this "last-resort antibiotic." The mechanisms of tigecycline resistance are highly diverse and include several known mechanisms, such as active efflux pump transport systems, mutations in the ribosomal protein binding site, drug degradation, and modification of the cell membrane (Fig. 6).

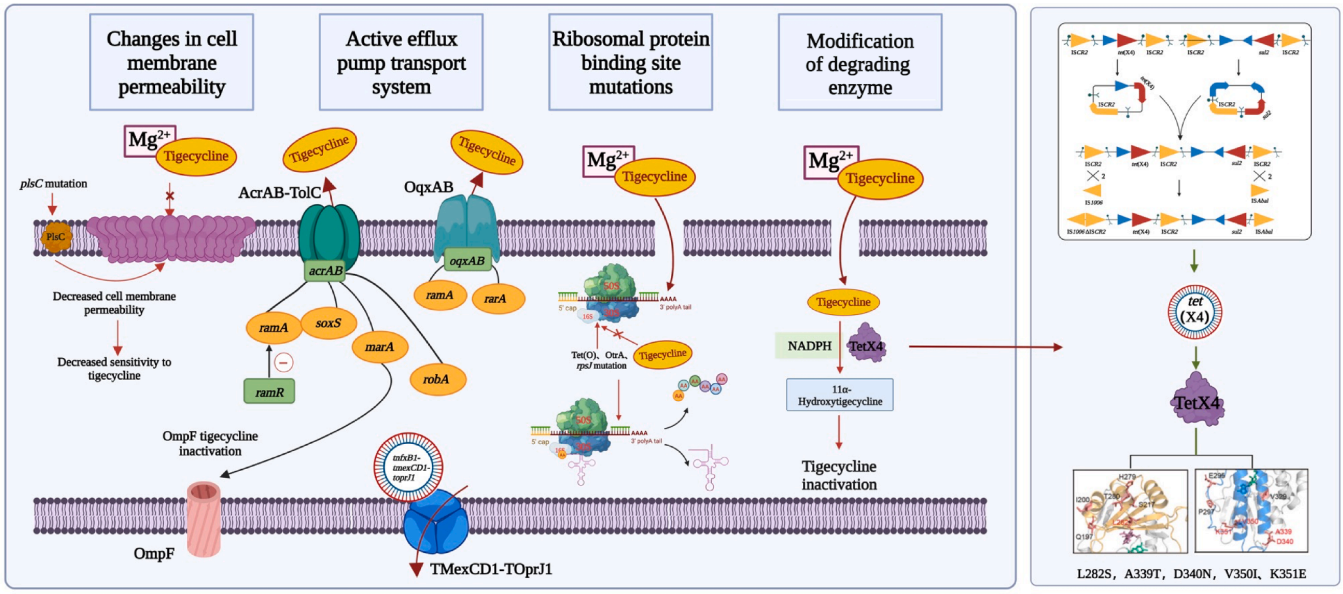

Fig. 6. Mechanisms of tigecycline resistance. The resistance mechanisms include mutation of ribosomal protein binding sites, modification of degrading enzyme, active efflux pump transport system, and changes in cell membrane permeability. The insertion sequence IS*CR2* plays a role in the horizontal transfer. Genetic recombination of the *tet*(X4) gene and sequence variations of five key residue (L282S, A339T, D340N, V350I, and K351E) lead to an increase in the activity of degrading enzyme Tet(X4) encoded by *tet*(X4). OmpF: Outer membrane protein F; AcrAB-TolC, OqxAB and TMexCD1-TOprJ1: Transmissible resistance nodulation-division (RND) family efflux pump; *plsC*: Membrane synthesis gene; *ramA, soxS, marA, robA, rarA*: Efflux pump regulator genes.

A recently identified plasmid-mediated RND family efflux pump, TMexCD1-TOprJ1, has been found to confer resistance to tigecycline and eravacycline. The Mex family proteins were initially discovered in the chromosomal genome of *P. aeruginosa*, providing intrinsic resistance to tetracycline, chloramphenicol, and norfloxacin. The *tmexCD1-toprJ1* represents the first plasmid-borne RND-type determinant that confers resistance to these antibiotics. It consists of the *tnfxB1-tmexCD1-toprJ1* operon architecture identified on a plasmid from a multidrug-resistant *K. pneumoniae* isolate [70]. The components of this efflux pump, TMexC1, TMexD1, and TOprJ1, exhibit similar secondary structures to other well-characterized RND efflux pump constituents, such as MexCD-OprJ from *P. aeruginosa* PAO1 and AcrAB-TolC from *E. coli* strain K-12 [70]. The *tnfxB1-tmexCD1-toprJ1* cluster is located within the transposon Tn*5393*, which includes two genes encoding a site-specific integrase (*int*) and two genes with unknown functions (designated as *hp1* and *hp2*, respectively). The site-specific integrase plays a role in capturing and co-transferring large DNA fragments, potentially facilitating the transfer and integration of *tnfxB1-tmexCD1-toprJ1* and its variants from the chromosome of *Pseudomonas* spp. into plasmids [71]. The presence of IS*26* in multidrug-resistant plasmids is commonly observed and likely contributes to the rapid and widespread transmission of *tmexCD1-toprJ1* in *Enterobacteriaceae* [71]. The *tmexCD1-toprJ1*, along with adjacent mobile elements, not only confers antibiotic resistance but also co-transfers alongside other antimicrobial genes, such as the polymyxin resistance gene *mcr-8.1* [71].

Moreover, mutations in the *tet*(A) gene, which encodes a plasmid-borne tetracycline efflux pump, have been reported to contribute to tigecycline resistance. Recent discoveries in China have highlighted the presence of plasmid-borne genes *tet*(X3), *tet*(X4), *and tet*(X5) that encode tigecycline-modifying enzymes in *A. baumannii* and *Enterobacteriaceae* [72,73]. Tet(X), encoded by *tet*(X), is a flavin-dependent monooxygenase that inactivates tetracycline by selectively adding hydroxyl groups. Through structural domain swapping and site-directed mutagenesis, a set of five key residue changes (L282S, A339T, D340N, V350I, and K351E) have been identified, which result in increased Tet(X) activity. These amino acid residues are conserved among all tigecycline-inactivation Tet(X) families, suggesting their crucial role in high-level tigecycline resistance.

Tet(X3) and Tet(X4) have been found to inactivate all tetracyclines, including newly approved antibiotics such as eravacycline and omadacycline. Plasmids carrying *tet*(X4) predominantly belong to incompatibility groups such as IncFIA-IncHI1B-IncHI1A, IncFIA-IncFIB-IncX1, IncFII, ColE2, IncQ, IncX1, and IncFIA-IncFIB-IncX1-IncN. Furthermore, *tet*(X4) has been found in the context of various mobile genetic elements, often adjacent to two copies of IS*CR2*, an IS*CR* element that shares 65% of its amino acid identities with IS*CR1* [74]. IS*CR2* has been identified in various plasmid backbones along with multiple resistance genes, including *floR*, *tetA*, *tetR*, *strA*, *strB*, *tet*(X4), and *sul2* [75]. The number of IS*CR2* elements increases with the presence of the *tet*(X4) gene, and circular intermediates containing IS*CR2* and *tet*(X4) have been detected, likely resulting in tandem duplications of *tet*(X4) [76]. Additionally, *tet*(X4) has been found adjacent to IS*Vsa3* in IncFIB-type plasmids. Similar regions with over 99% nucleotide sequence identity to IS*Vsa3* have been observed in more than 26 bacterial species worldwide [76]. The widespread occurrence of IS*Vsa3* elements and the formation or translocation of circular *ISVsa3-tet*(X) intermediates suggest the on-going spread of *tet*(X) variant. Notably, Tet (X)-positive *Bacteroides immobilis* isolates carrying bla_{NDM-1} or *mcr-1* have been found, indicating the emergence of *tet*(X) variants in polymyxin- or carbapenem-resistant bacteria [77].

4. Strategies against antibiotic resistance

As the global issue of antibiotic resistance continues to worsen, the need for innovative strategies becomes increasingly urgent. Researchers are undertaking epidemiological and genomic investigations to identify resistance factors and develop new adjuvant drugs that can enhance the efficacy of existing antibiotics. Examples include β-lactamase inhibitors, EPIs, and drugs targeting bacterial virulence proteins. Additionally, there is a critical need to identify and develop new antibiotic candidates that possess unique mechanisms of action, new scaffolds, and low potential for resistance.

Bioinformatics advancements play a crucial role in understanding resistance mechanisms by elucidating important hub genes/proteins and their interactions with key drug targets. Those knowledge is a vital step in the drug discovery [78]. Whole genome sequencing analysis and

proteomic analysis are effective in predicting resistance, with proteomics providing insight into the dynamic status of the cell [79]. Bioinformatics techniques have revealed multiple post-translational modifications (PTMs) in ESKAPE pathogens that regulate virulence and defense systems. These PTMs include the addition of chemical groups (such as acetylation, phosphorylation, methylation, and hydroxylation) or complex molecules (such as AMPylation, ADP-ribosylation, glycosylation, and isoprenylation) [79]. Bioinformatics also aids in the development of new drugs by predicting or modeling macromolecular structures and studying structure-function or structure-activity relationships (SAR) [80]. Protein dynamics simulations can assess protein stability, while virtual screening and prediction of pharmacokinetic/-pharmacodynamic (PK/PD) profiles can identify potential ligands. These advancements greatly facilitate drug design and repurposing efforts [81]. For example, using bioinformatics techniques, a series of thiophene derivatives as ligands were discovered for the histidine kinase, a key enzyme involved in PTMs, which contains a highly conserved adenosine triphosphate-binding domain [82]. Table 3 provides a brief overview of the principles and representative drugs of antibiotic synergists and new antibiotics.

4.1. Antibiotic synergists and resistance inhibitors

4.1.1. β-lactamase inhibitors

The production of β-lactamase is the most common and significant mechanism of β-lactam resistance in Gram-negative bacteria [83]. Currently, the combination of β-lactam antibiotics with β-lactamase inhibitors, such as clavulanate, sulbactam, and tazobactam, is an effective strategy to combat β-lactamase-mediated resistance [84]. However, the excessive use of antibiotics has led to selective pressure, accelerating the emergence of resistance to β-lactamase inhibitor combinations. To address this issue, a new generation of drugs is needed for combination therapy. Non-β-lactam β-lactamase inhibitors called diazabicyclooctanes have been developed, based on a (5 R)−7-oxo-1, 6-diazabicyclo[3.2.1]octan-6-yl sulphate core [85]. Another example is avibactam, which has a bicyclic core structure and can reversibly bind to the active site of serine β-lactamases [86]. Avibactam has been successfully developed as a potent inhibitor of class A and C β-lactamases and is clinically approved for the treatment of abdominal and UTIs and pneumonia [87]. While some clinical success has been achieved with combinations of β-lactams and β-lactamase inhibitors, the clinical solution for metallo-β-lactamases (MβLs) remains unclear [88]. Most MβL inhibitors target the zinc ions within the active sites of MβLs, aiming to disrupt the formation of ternary complexes or strip the metal ions [89]. Aspergillomarasmine A, an inhibitor of MβLs NDM-1 and VIM-2, acts by

Table 3
Strategies against antibiotic resistance.

Strategies		Mechanisms	Examples	Advantages	Limitations	Ref.
Antobiotic synergists	β-lactamase inhibitors	Reversing the active site of serine β-lactamases in a reversible manner	Avibactam			[86]
		Inhibiting the ativity of MβLs by chelating and removing the active site zinc ions	Aspergillomarasmine A			[90]
		Exploiting substrate mimicry as a tetrahedral intermediate	Taniborbactam			[91]
	Efflux pump inhibitors	Interference with the assembly of the discharge pump; Block the energy source of the efflux pump	CCCP; 1,3,4-oxadiazole	Universal	Cytotoxicity and poor pharmacokinetic properties	[96, 97]
	Membrane permeability enhancer	Binding to both LPS and phosphatidylglycerol to cause membrane damage	SLAP-S25			[98]
New antibiotics acting on different targets	New Quinolone agents	Binding to new site on the topoisomerase IV	Quinazolinediones			[99]
	Virulence protein-targeted antibiotics	Inhibiting the synthesis or binding of virulence factors, like Caseinolytic proteins, GroES/GroEL	D3, E2 and G2; Sulfonamido-2-arylbenzoxazole	Anti-biofilm effects and reducing the development of resistance.	Moderate or low cytotoxicity	[104, 105]
Biological alternative therapies	AMPs	Attraction (electrostatic bonding), attachment (joining with the LPS or teichoic or lipoteichoic acid), and peptide insertion	Lactoferricin B-Mutant M4; AuNPs@Esc (1–21)	High efficiency		[115, 116]
	Bacteriophage Therapy	Endolysins encodedby the bacteriophage genome digested the bacterial cell wall;	OMKO1; ECA2	high host specificity and low dosages for treatment	inducing overproduction of inflammatory mediators and coagulation factors; exchange of virulence or antibiotic resistance genes	[122, 128]
New carriers and antibacterial systems	Antimicrobial NPs	Strong synergistic antibacterial effects with antibiotics or peptides and increasing the local concentration of agents	Cobaltocene-containing methacrylate monomers polymerization silica NPs	Easy to modification, highly durable, stable, and low cytotoxicity		[112, 113]
Physical and chemical therapies	NTP	Damage of nucleic acids under UV radiation, lipid peroxidation, and the chemical modification and degradation of proteins by ROS	Plasma-activated water	Anti-biofilm effects and reducing the development of resistance.		[131]
	aPDI	Formation of excited triplet state state and damage to pathogens' nucleic acids, proteins, and lipids	aBL of 400-nm	Anti-biofilm effects and reducing the development of resistance.		[134]

MβLs: Metallo-β-lactamases; CCCP: Carbonyl cyanide m-chlorophenylhydrazone; SLAP-S25: Short linear antibacterial peptide-S25; D3, E2 and G2: Several trans-β-lactone compounds; AMPs: Antimicrobial Peptides; NPs: nanoparticles; Esc (1–21): Frog skin AMP esculentin-1a, esculentin-1a(1–21)NH2; AuNPs@Esc (1–21): covalent conjugation of Esc(1–21) to soluble AuNPs; OMKO1: a lytic bacteriophage of P. aeruginosa; ECA2: a bacteriophage of E. coli; NTP: Non-thermal plasma; UV: Ultraviolet; ROS: Reactive oxygen species; aPDI: Antimicrobial photodynamic inactivation; aBL: Antimicrobial blue light

chelating and removing the active site zinc ions, thereby resensitizing *Pseudomonas* spp., Acinetobacter spp., and Enterobacteriaceae MβL-producing strains to meropenem [90]. Currently, taniborbactam (VNRX-5133) is undergoing phase 3 clinical testing and is expected to be developed as a new cycloborate β-lactamase inhibitor [91].

4.1.2. Efflux pump inhibitors (EPIs)

Efflux pumps play a significant role in both intrinsic and acquired bacterial resistance [92]. Therefore, EPIs represent promising strategies for combating antibiotic resistance. However, several challenges need to be addressed before EPIs can be widely used. Firstly, efflux pumps can undergo mutations to restrict the applicability of EPIs [93]. Secondly, EPIs often exhibit toxic effects and have poor pharmacokinetic properties. For instance, peptidomimetic PAβN, pyridopyrimidinone, pyranopyridine, and derivatives of quinoline and indole have not been approved for therapeutic use due to these limitations [94].

Nevertheless, collective efforts are being made to identify potential EPIs. For example, innamamide, which combines carbamate and indol-3-yl groups, has shown the most activity and acceptable toxicity against human lung fibroblast MRC5 cells [95]. Carbonyl cyanide m-chlorophenylhydrazone (CCCP) has demonstrated the ability to reverse polymyxins resistance in various bacterial strains [96]. Additionally, 1,3,4-oxadiazole has been identified as a potent inhibitor of the NorA efflux pump [97]. These examples illustrate ongoing research to screen and develop effective EPIs. Overcoming the challenges associated with toxicity and pharmacokinetics will be crucial for the successful application of EPIs as a strategy to combat antibiotic resistance.

4.1.3. Membrane permeability enhancer

Another strategy to enhance the activity of antibiotics is by disrupting the outer membrane or increasing its permeability. A recent discovery in this field is SLAP-S25, a novel linear short-chain broad-spectrum antibacterial synergist. SLAP-S25 can tightly bind to bacterial lipopolysaccharides (LPS), even in the presence of modifications mediated by MCR enzymes [98]. Specifically, SLAP-S25 recognizes the phosphate radical of the head group of phosphatidylglycerol, leading to an increase in the permeability of the bacterial membrane.

This approach of enhancing membrane permeability offers a potential avenue for improving the effectiveness of antibiotics by allowing them to penetrate bacterial cells more efficiently. Further research and development in this area may lead to the discovery of additional membrane permeability enhancers that can enhance the activity of antibiotics against resistant bacteria.

4.1.4. Change action site

Quinolones target DNA gyrase and topoisomerase IV enzymes. Mutations in the QRDR of these enzymes disrupt the water-metal ion bridge, leading to reduced susceptibility to quinolones. Recent studies have focused on finding new binding sites on these enzymes to overcome quinolone resistance. Quinazolinediones, which have a similar structure to quinolones, replace the keto acid moiety with an R2 carbonyl group that can bind to a conserved arginine residue on the enzyme through a hydrogen bond. This alternative binding site appears to overcome the resistance that has developed against quinolones [99]. Furthermore, combining quinolones with other antibacterial pharmacophores has emerged as a promising strategy to develop safe and highly effective antibacterial agents. This approach targets multiple bacterial targets and leverages synergistic effects [100]. For example, 1,2,4-triazole-quinoline/quinolone hybrids have shown antibacterial activity that is 2–32 times higher than that of ciprofloxacin, a widely used quinolone antibiotic [101].

By exploring alternative binding sites and combining quinolones with other antibacterial agents, researchers aim to develop new compounds that can overcome quinolone resistance and provide improved efficacy against resistant bacteria. These approaches offer potential solutions to combat the challenge of antibiotic resistance.

4.1.5. Virulence protein-targeted antibiotics

In addition to directly killing bacteria, targeting virulence factors is an effective approach to prevent bacterial pathogenesis [102]. Currently, various members of the ClpA and ClpB/Hsp104 subfamilies have been identified in the ESKAPE pathogens [102]. Caseinolytic proteins play essential roles in intracellular protein homeostasis and stress response [103]. Compounds such as D3, E2, and G2 have been found to irreversibly inhibit the proteolytic activity of ClpP in *S. aureus* [104]. Sulfonamido-2-aryl benzoxazole has been shown to inhibit the GroES/GroEL chaperone system [105]. However, many of these compounds exhibit moderate or low cytotoxicity to human liver and kidney cells. A new model has been developed using *E. coli* to target the chaperone protein GroES/GroEL of ESKAPE pathogens [106]. This approach offers potential for the development of novel drugs that specifically target bacterial chaperone proteins. Additionally, compounds like Calystegine B3, 7,7 A-diepialexine, and α-ethyl noradrenaline have been found to regulate biofilm development and formation by binding to the *A. baumannii* BfmR (RstA) protein [107].

By targeting virulence factors, researchers aim to disrupt the pathogenicity of bacteria and prevent the development of infections. Developing drugs that specifically target these factors can provide alternative strategies to combat bacterial infections and reduce their impact on human health.

5. Alternative therapies of non-antibiotics

5.1. Antimicrobial nanoparticles

Antimicrobial nanoparticles (NPs) have emerged as promising alternatives to traditional antibiotics. Various types of NPs, such as silver, gold, zinc oxide, titanium oxide, and silica, have shown strong antibacterial effects and synergistic activity when combined with antibiotics or peptides. Metal oxide NPs (MO-NPs) are particularly attractive due to their durability, stability, and low cytotoxicity. Gold NPs (GNPs) have received FDA safety approval and offer advantages such as small size, large surface area, easy modification, and resistance to bacterial efflux pumps [108].

Combining antibiotics with GNPs has been shown to enhance bactericidal activity against both Gram-positive and Gram-negative bacteria while reducing cytotoxicity. Cationic metallo-polymers have also been developed to enhance the activity of penicillin G and effectively inactivate bacteria [109]. Silver NPs (AgNPs) have demonstrated synergistic effects with antibiotics such as ampicillin, chloramphenicol, and kanamycin against various bacterial strains, including multidrug-resistant ones [110]. Additionally, the combination of Au:Ag alloy NPs with antibiotics has shown stronger antibacterial effects compared to using AuNPs or AgNPs alone, integrating the benefits of both materials [111].

Functionalizing metal-based nanomaterials with additional components can further enhance their antimicrobial activity. Conjugating porphyrin-AgNPs with light-triggered photodynamic effects have demonstrated higher antibacterial activity compared to pristine AgNPs [112]. Hybrid materials composed of humic acid and titanium dioxide nanomaterials have shown excellent antibacterial activity against various strains, overcoming the limitations of HA's selectivity and stability. Multifunctional nanocellulose-based hybrid materials incorporating metals or metal oxides have also shown potential in treating multidrug-resistant bacterial infections. For instance, alginate-zinc oxide nanomaterials have exhibited higher antibacterial activity against MRSA with lower cytotoxicity compared to zinc oxide or chitosan-zinc oxide [113].

These antimicrobial NPs offer a novel approach to combating bacterial infections and also can be used in combination with traditional antibiotics to enhance their efficacy. The incorporation of functional components and hybridization of nanomaterials further expand the possibilities for developing effective therapies against multidrug-

resistant bacteria. New carriers and antimicrobial systems based on nanometers can be seen in Table 3.

5.2. Antimicrobial peptides (AMPs) therapy

AMPs therapy is a traditional alternative to combat pathogen resistance. AMPs exert their antibacterial effects through attraction (electrostatic bonding), attachment (binding to lipopolysaccharides, teichoic acids, or lipoteichoic acids), and peptide insertion into bacterial cells [114]. Lactoferricin B-Mutant has demonstrated high affinity against various resistant bacteria, including SHV1, OXA48, NDM1, and AmpC [115]. However, bacteria have developed resistance to AMPs by altering their cell surface or releasing proteolytic enzymes, highlighting the need for new delivery systems.

One approach involves covalently conjugating antimicrobial peptides, such as ESC (1−21), to PEGylated gold nanoparticles (AuNPs@PEG) to form [AuNPs@Esc (1−21)] [116]. This conjugation allows the peptide to maintain its antibacterial activity even in the presence of proteolytic enzymes and trypsin. Another strategy is the use of "structurally nano-engineered" antimicrobial peptide polymers (SNAPPs) [117]. These polymers, developed by Lam et al., exhibit sub-μM activity against ESKAPE pathogens, including colistin-resistant and MDR strains. SNAPPs enhance membrane instability, disrupt ion movement across the cytoplasmic membrane, and induce an apoptosis-like cell death pathway in the bacteria [118].

By harnessing the antimicrobial properties of AMPs and developing innovative delivery systems, AMPs therapy holds promise as an alternative approach to combat antibiotic-resistant bacteria. AMPs offer a unique mechanism of action and can target a broad spectrum of pathogens, making them attractive candidates for future antimicrobial therapies.

5.3. Bacteriophage therapy

Bacteriophage therapy offers several advantages, including high host specificity, low dosages for treatment, and rapid proliferation inside the host bacteria [119]. Bacteriophages mutate alongside their host, allowing them to develop new infectivity and gain an advantage over bacteria [120]. T. The primary mechanism of action for bacteriophages against ESKAPE pathogens is bacterial lysis, facilitated by endolysins encoded by the bacteriophage genome that digest the bacterial cell wall and release offspring bacteriophages [121]. For example, the lytic bacteriophage OMKO1 targets the OprM of the multidrug efflux systems MexAB and MexXY in *P. aeruginosa* [122].

However, there are limitations to bacteriophage therapy. The overproduction of inflammatory mediators and coagulation factors induced by massive bacterial lysis may lead to septic shock, blood vessel damage, intravascular coagulation, and organ failure [121]. Bacteriophages can also potentially participate in the exchange of virulence or antibiotic-resistance genes, which may make pathogens more pathogenic or resistant to antibiotics [123–125]. Nevertheless, extensive research has been conducted to identify ideal bacteriophages for therapeutic purposes, and the biosafety of bacteriophage therapy should be verified [126]. It is crucial to thoroughly investigate the choice of phage type, antibiotic combination, and their mixing ratios to optimize the antibacterial approach [127]. For instance, the combination of ECA2, a phage that acts on *E. coli*, with ciprofloxacin has shown highly synergistic effects [128].

Overall, bacteriophage therapy holds promise as a potential alternative to combat antibiotic-resistant bacteria. Further research and understanding of its mechanisms, safety, and optimal utilization are necessary to harness its full potential in clinical applications.

5.4. Non-thermal plasma (NTP)

NTP is a specialized form of plasma that is partially ionized, meaning it contains a mixture of charged particles and free electrons. Unlike thermal plasmas, NTP does not rely on high temperatures to achieve ionization. Instead, the energy in NTP is primarily stored in the free electrons present in the gas.

NTP has gained attention for its antimicrobial and anti-biofilm properties. It has been found to be effective against a wide range of microorganisms, including bacteria, viruses, and fungi. One of the notable advantages of using NTP as an antimicrobial treatment is its reduced potential for promoting the development of antibiotic resistance in pathogens. This is in contrast to traditional antibiotic treatments, which can contribute to the emergence of drug-resistant strains [129]. The mechanism by which NTP exerts its antimicrobial effects is multifaceted. When NTP is applied to microorganisms, several processes occur simultaneously. The intense UV radiation emitted by NTP can damage the genetic material of microorganisms, such as their nucleic acids, leading to cell death. Additionally, the generation of reactive oxygen species during NTP exposure can cause oxidative stress and damage to the lipids and proteins of microorganisms. Lipid peroxidation, for example, disrupts the integrity of microbial cell membranes, compromising their survival [130].

Another intriguing application of NTP is the generation of plasma-activated water (PAW). PAW is produced by treating water with NTP, leading to the formation of reactive species and the transfer of plasma-generated agents into the liquid. PAW has been shown to possess long-lasting bactericidal activity, maintaining its effectiveness for several months. This makes PAW a potentially valuable tool for disinfection and sanitation purposes, particularly in healthcare settings and food processing industries [131].

Overall, NTP represents a promising approach for combating microbial infections and biofilm-related issues. Its unique properties and mechanisms of action make it an attractive alternative to traditional antimicrobial treatments, offering potential benefits such as reduced resistance development and long-lasting efficacy. However, further research is still needed to optimize NTP-based technologies and understand their full potential in various applications.

5.5. Antimicrobial photodynamic inactivation (aPDI)

APDI is a technique that utilizes a process called photodynamic therapy to destroy microorganisms. It involves the use of a photosensitizer (PS), which is a chemical compound that can absorb light energy. The PS is typically administered to the target area, such as the site of infection or biofilm formation.

When the PS is exposed to light of an appropriate wavelength, it becomes excited and enters an energized state. This excited state can lead to the formation of a reactive species called singlet oxygen, which is highly reactive and can cause damage to the microorganisms. The singlet oxygen can attack various components of the microorganisms, including their nucleic acids (DNA and RNA), proteins, and lipids, leading to their destruction [132,133]. Additionally, a specific type of light called blue light (aBL) with a wavelength of around 400 nm has been found to have broad-spectrum antibacterial and anti-biofilm activity against a group of pathogens known as the ESKAPE pathogens [134]. The aBL can directly affect these pathogens, disrupting their cellular structures and functions, and ultimately leading to their inactivation.

Antimicrobial photodynamic inactivation offers a targeting, non-invasive and potentially effective approach for the treatment of various microorganisms and biofilm-related infections, including those that may be resistant to traditional antibiotics. However, the specific photosensitizers, light parameters, and treatment protocols need to be carefully optimized for different microbial targets and clinical applications. Further research and clinical studies are necessary to fully understand and harness the potential of aPDI in the field of antimicrobial therapy. A brief list of biological alternative therapies (AMPs and bacteriophages) and physical-chemical therapies (NTP and aPDI) are

presented in Table 3.

6. Future perspectives and directions

Although there is a global consensus on limiting the use of antibiotics, the critical situation of antibiotic resistance persists. The increasing emergence of antibiotic-resistant bacteria and the scarcity of novel available antibiotics present significant challenges for global healthcare systems. It is evident that novel antibiotic-resistant bacteria rapidly emerge through the plasmid-driven horizontal transmission of mobile resistance genes, such as *mcr, bla*$_{KPC}$, *tmexCD-toprJ,* and *tet*(X4). The coexistence of various resistance mechanisms within a single strain poses a major threat to public health, such as ESKAPE pathogens. Antibiotic resistance genes are disseminated widely through specific mobile elements like IS or integrons. For instance, transmissible multidrug IncFIB/IncHI1B plasmids carrying *tmexCD2-toprJ2*, *bla*$_{NDM-4}$, and *bla*$_{NDM-1}$ have been reported to confer resistance to both tigecycline and carbapenems [135]. To address these challenges and combat antibiotic resistance, future research, and directions should focus on the following:

- Understanding Resistance Mechanisms: Efforts should be directed towards gaining a comprehensive understanding of the mechanisms underlying resistance to antibiotics like polymyxins, carbapenems, and tigecycline. This involves studying the modification of lipid A of LPS catalyzed by MCR enzymes encoded by genes like *mcr-1* to *mcr-10* in polymyxin resistance. For CR, the production of antibiotic hydrolases (such as carbapenemases), alteration of bacterial outer membrane permeability, drug efflux pumps, and modification of drug targets like PBPs should be explored. Tigecycline resistance mechanisms, including drug efflux pumps, ribosomal protein binding site mutations, changes in cell membrane permeability, and tigecycline-modifying enzymes encoded by *tet*(X) variants, require further investigation.
- Novel Therapeutic Strategies: Developing innovative therapeutic approaches is crucial to overcome antibiotic resistance. Future research should focus on the discovery and development of new antimicrobial agents that can effectively target and combat multidrug-resistant bacteria. This may involve exploring alternative treatment modalities such as NTP and aPDI as potential adjunct therapies.
- Combination Therapies and Synergistic Approaches: Investigating combination therapies and synergistic approaches involving multiple antimicrobial agents can be a valuable strategy to address multidrug-resistant bacterial infections. Research efforts should be directed toward identifying optimal combinations that can enhance efficacy, overcome resistance mechanisms, and minimize the emergence of further resistance.
- Infection Control Measures and Antibiotic Stewardship: Strengthening infection control measures is crucial to prevent the spread of multidrug-resistant bacteria. Promoting effective hygiene practices, implementing surveillance systems, and developing antimicrobial materials for medical devices and surfaces can contribute to infection prevention. Additionally, antibiotic stewardship programs should be reinforced to ensure appropriate antibiotic use, reduce unnecessary prescriptions, and mitigate the selective pressure that drives antibiotic resistance.

Addressing the challenges posed by antibiotic resistance requires a multi-faceted approach. Future perspectives and directions should involve a comprehensive understanding of resistance mechanisms, the development of novel therapeutic strategies, exploration of combination therapies, the implementation of infection control measures, and the promotion of antibiotic stewardship. By focusing on these areas, we can make significant progress in combating antibiotic resistance and improving the management of multidrug-resistant bacterial infections in biomedical settings.

7. Conclusions

In conclusion, the global challenge of antibiotic resistance remains a critical issue in healthcare systems worldwide. The emergence of antibiotic-resistant bacteria, coupled with the limited availability of new antibiotics, highlights the urgent need for alternative strategies. The horizontal transmission of mobile resistance genes and the presence of multiple resistance mechanisms in a single strain further complicate the situation. Efforts to address antibiotic resistance have explored novel approaches such as broad-spectrum antibacterial synergists and antimicrobial nanoparticles. These advancements have shown the potential in enhancing the effectiveness of existing antibiotics and improving their efficacy against multidrug-resistant bacteria. Additionally, addressing biofilm formation and understanding the mechanisms underlying pathogen virulence and resistance is essential for developing targeted therapies. To combat antibiotic resistance effectively, future research should focus on optimizing broad-spectrum synergists and nanoparticles, exploring anti-biofilm strategies, and gaining further insights into pathogenvirulence determinants and aggravating factors. By doing so, scientific community can develop innovative antimicrobial agents, preventive measures, and combinatorial therapies contributing to fighting against multidrug-resistant bacterial infections.

Author contributions

QW, JT, and AZ coordinated the writing of the manuscript. SI, AZ, YZ, LD, SD, JL, and QW participated in drafting and editing the text and figures. All authors read and gave final approval to the version submitted.

Ethics approval and consent to participate

Not applicable.

Funding

This work was supported by the Zhejiang Provincial Natural Science Foundation of China (LQ20H190004), the Key R&D Program of Zhejiang (2022C03060), and the Double Thousand Talents Plan of Jiangxi (jxsq2019101054), and the National Natural Science Foundation of China (32260032 & 31800118 & 81401710).

CRediT authorship contribution statement

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

We thank the Elixigen Corporation for reading our manuscript and providing native English professional support.

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